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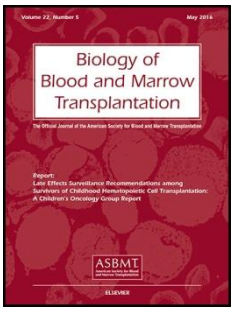

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Immunomodulatory effects of tyrosine kinase inhibitors (TKIs) *in vitro* and *in vivo* study.

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Short title:

In vitro and *in vivo* effects of TKIs on lymphocytes

Keywords:

Tyrosine kinase inhibitors (TKIs); Chronic Graft Versus Host Disease (cGVHD); Lymphocyte subpopulations; T regulatory cells; Cytokine production.

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Highlights:

- TKIs suppress the production of pro-inflammatory cytokines in vitro, including IL17
- TKIs reduce the frequency of total Tregs *in vitro* but preserve naïve subsets
- B and NK cells are impaired by TKIs in vitro, whereas T cells are preserved
- Comparable results were obtained in patients treated with Nilotinib for cGVHD

Abstract

Pathogenesis of chronic Graft Versus Host Disease (cGVHD) is incompletely defined, involving donor-derived CD4 and CD8-positive T lymphocytes, as well as B cells. Standard treatment is lacking for steroid dependent/refractory cases; potential usefulness of tyrosine kinase inhibitors (TKIs) has been suggested, based on their potent anti-fibrotic effect. However, TKI seems to have pleiotropic activity. We sought to evaluate the *in vitro* and *in vivo* impact of different TKIs on lymphocyte phenotype and function. Peripheral blood mononuclear cells (PBMC) from healthy donors were cultured in presence of increasing concentrations of Nilotinib, Imatinib, Dasatinib and Ponatinib; in parallel 44 PBMC samples from 15 patients with steroid-dependent/refractory cGVHD treated with Nilotinib in the setting of a phase 1-2 trial were analyzed at baseline, after 90 and after 180 days of therapy. Flow cytometry was performed after labelling lymphocytes with a panel of monoclonal antibodies (CD3, CD4, CD16, CD56, CD25, CD19, CD45RA, FoxP3, CD127 and 7AAD). Cytokine production was assessed in supernatants of purified CD3+ T cells and in plasma samples from Nilotinib-treated patients. Main T lymphocytes subpopulations were not significantly affected by therapeutic concentrations of TKIs *in vitro*, whereas pro-inflammatory cytokine (in particular IL2, IFNγ, TNFα and IL10) and IL17 production showed a sharp decline. Frequency of T regulatory, B and NK cells decreased progressively in presence of therapeutic

concentrations of all the TKIs tested *in vitro*, except for Nilotinib that showed little effect on these subsets; of note, naïve T regulatory cell subset accumulated following exposure to TKIs. Results obtained *in vivo* on Nilotinib-treated patients were largely comparable, both on lymphocyte subset kinetics and on cytokine production by CD3-positive cells. This study underlines the anti-inflammatory and immunomodulatory effects of TKIs, and support their potential usefulness as treatment for patients with steroid dependent/refractory cGVHD. In addition, both *in vitro* and *in vivo* data point out that, compared to other TKIs, Nilotinib could better preserve the integrity of some important regulatory subsets, such as T regulatory and NK cells.

Introduction

Chronic Graft Versus Host Disease (cGVHD) is a major complication of allogeneic stem cell transplantation (allo-SCT) and is characterized by multi-organ involvement resembling autoimmune diseases. The pathogenesis of cGVHD has not been fully elucidated [1]; however recent insights suggest that several players and different pathways are involved, including imbalance of T and B cells, and exaggerated collagen production; these phenomena are associated with typical alterations of the cytokine network, such as increased levels of transforming growth factor beta (TGFβ) and inflammatory cytokines. Similarly to what observed in systemic autoimmune diseases, a variety of autoantibodies have been reported in patients affected by cGVHD [2,3] and many of them develop multi-organ disease with fibrotic features, resembling systemic scleroderma (SSc); typical biological data are characterized by increased production of pro-fibrotic inflammatory cytokines such as platelet-derived growth factor (PDGF) and TGFβ [4,5]. Perturbation of regulatory T cell (Treg) homeostasis has also been reported as a contributing factor to both acute and cGVHD [6,7]. Moreover, B lymphocytes have recently been recognized as important players in the pathogenesis of cGVHD [8,9]; indeed, relevant clinical response rates

have been reported following Rituximab-induced B cell depletion [10]. In general, mild cGVHD only requires topic treatment and ancillary therapeutic measures; on the other hands, both moderate and severe cGVHD, defined according to the National Institute of Health consensus criteria, require systemic treatment [11]. The standard first-line systemic treatment of cGVHD consists of steroids with or without calcineurin inhibitors, yielding a response rate of 50-60%, [12-15]; however, the optimal treatment for steroid refractory cGVHD (sr-cGVHD) has still to be defined [16] and long-term steroid administration in steroid-dependent patients is associated with severe chronic toxicity. Imatinib, a first-generation Tyrosine Kinase Inhibitor (TKI) is currently used for treatment of Philadelphia-positive chronic myelogenous leukemia (CML) and acute lymphoblastic leukemia (ALL). This drug strongly inhibits both PDGF and TGFβ pathways *in vitro*; moreover, increasing concentrations of Imatinib prevent up-regulation of the major extracellular matrix (ECM) proteins COL1A1, COL1A2, and fibronectin 1 in PDGFβ and TGFβ-stimulated dermal fibroblasts. [17] Three prospective clinical trials suggest the potential usefulness of Imatinib in patients with sr-cGVHD [18-20]. In addition, preliminary data suggest that second-generation TKIs Nilotinib and Dasatinib could also be effective and well tolerated in patients with sr-cGVHD [21-22]. In the few years, a pleiotropic anti-fibrotic and immune-modulating activity of these drugs has been reported [23-25]; these drugs interfere with tyrosine kinase-mediated intracellular pathways and inhibit TCR-mediated signal transduction, cellular proliferation, cytokine production, and T-cell responses [26]. Moreover some *in vitro* data suggest that Treg function can be hampered by therapeutic concentrations of Imatinib in CML patients [27]. Nevertheless, the interactions between TKIs and the different immune subpopulations have not been fully elucidated. To better define the potential role of TKIs as therapeutic agents in cGVHD, we investigated the *in vitro* effect of four different drugs (i.e Nilotinib, Dasatinib, Imatinib, Ponatinib) on T, B and NK lymphocyte

subpopulations; data obtained *in vitro* were subsequently correlated with data obtained from a cohort of patients receiving Nilotinib for sr-cGVHD.

Materials and Methods

Samples for *in vitro* experiments

Peripheral blood samples were obtained after informed consent from residual unused blood collected from twenty healthy donors. Peripheral blood mononuclear cells (PBMC) were purified by density gradient centrifugation using Ficoll-Biocoll separation solution (*MP Biomediacals,* Santa Ana, California). Lymphocyte subpopulation frequencies were assessed by flow cytometry following culture, as described below.

Samples for *in vivo* experiments

PBMC and plasma samples were collected from patients participating to the Prospective, phase I/II, multicenter study to determine safety and efficacy of Nilotinib in a population with steroid-refractory/or steroid-dependent cGVHD (EUDRACT: 2010-023068-41). The study was designed to assess efficacy, safety and tolerability of four different doses of Nilotinib (200, 300, 400 and 600 mg/day) in patients affected by steroid refractory or steroid-dependent cGVHD. Samples were collected at baseline, after 90 and after 180 days of treatment; PBMC and plasma were subsequently frozen and stored at -80 °C; both lymphocyte subpopulation frequencies and cytokine production were assessed immediately after thawing (see below).

Cell isolation and cultures

CD3-positive T cells were purified from peripheral blood through positive selection by immune-magnetic cell separation with MACS CD3 microbeads (*Miltenyi Biotec*, Cologne, Germany),

according to the manufacturer's instruction; purity level obtained by the procedure was ≥90%, as assessed by flow cytometry (see below). Cells were placed in wells at the concentration $1x10^6$ cell/well and cultured for 96 hours in RPMI 1640 (*EuroClone*, Milan, Italy) supplemented with heat-inactivated 10% fetal bovine serum (*EuroClone*), 2 mM L-glutamine and 100 units/ml penicillin-streptomycin (*EuroClone*).

Incubation with tyrosine kinase inhibitors

PBMC from healthy donors were cultured as previously described both in absence and in presence of increasing concentrations of tyrosine kinase inhibitors (TKIs) Nilotinib, Imatinib, Dasatinib and Ponatinib. TKIs for *in vitro* experiments were provided by Santa Cruz Biotechnology and stored in aliquots at -20°C as 10 mM stock solution in DMSO, protected from light until use. For culture purpose, stock solution was diluted by RPMI 1640 and added to T cell cultures at serial concentrations on the first day of culture. Final concentrations were the following: 1 µM, 10 µM, 50 µM for Imatinib; 0,5 µM, 1 µM, 2 µM, 10 µM for Nilotinib; 50 nM, 100 nM, 200 nM for Dasatinib; 10 nM, 50 nM, 100 nM for Ponatinib.

Flow cytometry

Six-colour flow cytometry analysis was performed by FacsCanto II (*Becton-Dickinson*, Franklin lakes, New Jersey) after labelling cultured or patients-derived PBMC with the following conjugated monoclonal antibodies (*Becton-Dickinson*): CD4*fluorescein isothiocyanate (FITC), CD16-CD56*phycoerythrin (PE), CD3* phycoerythrin-Cyanine 7 (PE-Cy7), CD25* Allophycocyanin (APC) CD19* Allophycocyanin-Cyanine 7 (APC-Cy7), CD45RA* phycoerythrin (PE), transcription factor forkhead box P3 (FoxP3)* Peridinin Chlorophyll Protein-Cyanine 5.5, (PerCP-Cy5.5), CD127*phycoerythrin (PE), CD3* Allophycocyanin-Cyanine 7 (APC-Cy7), 7-Aminoactinomycin D

(7AAD). CD8 T cells were identified as CD3-positive and CD4-negative lymphocytes: to minimize possible overestimation, due to the presence of CD4- and CD8-double negative T cells, CD3-positive cells with very high mean fluorescence intensity (mainly represented by the double negative T cells) were gated out if present. For extracellular staining, cells were incubated for 30 min at room temperature with optimal dilution of each antibody, according to the manufacturer instructions. For intracellular staining, cells were fixed with Reagent A following surface labelling, then permeabilized with Reagent B (*Becton-Dickinson*) before intracellular staining. A minimum of 50.000 cells per tube were acquired; frequencies of the different subpopulations were subsequently calculated by FacsDiva software (*Becton-Dickinson*). To analyze the effects of TKIs on regulatory T cells, CD3-positive and CD8-negative lymphocytes (CD4-positive T cells) were gated, and regulatory T cells were subsequently identified as CD25-positive, FOXP3-positive and CD127 low or negative cells.

Cytokine output *in vitro* and *in vivo*

Purified cells were cultured as previously described; after 96 hours incubation with TKIs, supernatants were collected and cytokine concentrations were determined by Bio-Plex (*Bio-Rad*, Hercules, California), according to the instruction of Bio-Plex Pro Human Cytokine 17-plex Assay (*Bio-Rad*). Plasma samples from Nilotinib-treated patients and purified CD3-positive cells from healthy donors were also analyzed after thawing. Cytokines included in the panel were the following: IL2, IL4, IL10, IL17, IFNγ, TNFα. The Bio-Plex immunoassays are formatted on magnetic beads, based on a principle like a sandwich ELISA. Coupled beads react with the sample containing the biomarker of interest. After a series of washes to remove unbound protein, a biotinylated detection antibody is added to create a sandwich complex. The final detection complex is formed

with the addition of streptavidin-phycoerythrin conjugate. This kind of assay is able to quantify multiple protein biomarkers in a single well of a 96-well plate.

Statistical analysis

Data were compared as mean ± standard deviation (SD). Differences were assessed by two-tailed Student's t-test or Pearson's test and were considered statistically significant when $p<0.05$.

Results

In vitro effects of tyrosine kinase inhibitors on lymphocyte subpopulations

PBMC from healthy donors were cultured in presence of increasing concentrations of different TKIs; lymphocytes were gated by forward scatter (FSC) and side scatter (SSC) profile. Subpopulations were identified on gated lymphocytes as CD3- and CD4- double positive (T helper cells), CD3-positive and CD4-negative (T cytotoxic cells); CD19-positive (B lymphocytes); CD16- and CD56-double positive (Natural Killer cells, NK). As shown in table 1, exposure to increasing concentration of Imatinib (1 to 50 μM), Nilotinib (0.5 to 10 μM), Dasatinib (up to 100 nM) and Ponatinib (up to 50 nM) did not significantly affect the proportions of T cells. However, very high concentration of Dasatinib (200 nM) and Ponatinib (100nM) induced a sharp and profound decrease of total T lymphocytes (from 75.9% to 0% for both drugs, $p<0.02$), associated with a proportional increase of T cell death rate (from 0.1% to 100% for both drugs, $p<0.02$). This effect was observed both for T helper CD4 and for cytotoxic CD8-positive T cell subsets (supplementary figure S1 and S2). Both B an NK cells showed a progressive and significant reduction after exposure to increasing concentrations of either Imatinib, Dasatinib and Ponatinib. As shown in table 1, B cells decreased from 9.1% to 7.4% (p= NS), 5.7% ($p<0.05$) and 4.6% ($p<0.02$) following incubation with 1μM, 10μM and 50μM of Imatinib, respectively; to 3.4% ($p<0.02$), 3.5% ($p<0.02$) and 0% ($p<0.02$) following 50nM, 100 nM and 200nM of Dasatinib respectively; to 3.5% ($p<0.02$), 1.7% ($p<0.02$) and 0% ($p<0.02$) following 10nM, 50nM and 100nM of Ponatinib, respectively. Similarly, NK cell frequencies were reduced from 7.2% to 6.2% (p=NS), 6,1% ($p<0.05$) and 2.5% ($p<0.05$) by Imatinib; to 3.2% ($p<0.02$), 2.4% ($p<0.02$) and 0% ($p<0.02$) by Dasatinib; to 5% ($p<0.02$), 1.9% ($p<0.02$) and 0% ($p<0.02$) by Ponatinib. All these changes were paralleled by proportional increases in B lymphocytes and NK cell death rates (table 1). On the contrary, increasing *in vitro* concentrations of Nilotinib did not exert any significant impact on these two subpopulations (table

1); compared to baseline, no significant changes were observed in the proportion of both B lymphocytes (9.1 vs 8.6% p=NS) and NK cells (7.2 vs 5.5% p=NS), even at the highest drug concentration (10 μM). Taken together, these data suggest that: 1) compared to B lymphocytes and NK cells, T lymphocytes are more resistant to the toxic effects of all the TKIs tested; 2) Nilotinib appears to be less toxic than the other TKIs tested, being the only drug not exerting any significant impact on B lymphocytes and NK subset.

In vitro effects of tyrosine kinase inhibitors on regulatory T cells

As shown in figure 1 (panels A, C-D), *in vitro* exposure to Imatinib, Dasatinib and Ponatinib induced a sharp and progressive decrease of regulatory T cells proportion, statistically significant starting from the lowest drug concentration in culture ($p<0.02$); a decrease of regulatory T cells was also induced by Nilotinib, but only at concentrations 2 μM or higher (Figure 1, panel B). The contribution of cell death to the decrease of regulatory T cells observed following exposure to TKIs was also investigated (Figure 1, panels E-H). A progressive increase of apoptotic regulatory T cells was observed after exposure to increasing concentrations of Dasatinib and Ponatinib (Figure 1, panels G-H). Regulatory T cell viability was also impaired by increasing concentration of Imatinib (Figure 1, panel E), albeit to a lesser extent; again, incubation with Nilotinib was followed by an increase of regulatory T cell apoptosis that was mild and only observed at the highest concentrations of the drug (Figure 1, panel F). Of note, the reduction of the proportion of regulatory T cells did not involve naïve subset (identified as CD45RA-positive Treg); in fact, exposure to increasing concentrations of TKIs (with the only exception of Dasatinib) was followed by accumulation of these cells (Figure 2, panels A-D). These *in vitro* data suggest that: 1) overall, exposure to TKIs induce a profound decline of total regulatory T cells; 2) Nilotinib constitutes an

exception, unlike the other TKI, this drug does not impair regulatory T cells frequencies at therapeutic concentrations; 3) the decline observed in total regulatory T cells does not involve naïve cells, whose frequency increase following exposure to all the TKIs tested.

In vitro effects of tyrosine kinase inhibitors on T cell cytokine production

To investigate how TKIs can influence cytokine production by T cells, sorted CD3-positive cells were incubated with increasing concentrations of Imatinib, Nilotinib, Dasatinib, and Ponatinib; after 96-hour culture, supernatants were collected and cytokine levels were measured (Figure 3). Increasing concentrations of all the tested TKIs induced a consistent reduction of IL17 levels in culture supernatants compared to baseline (31.5±18.3 pg/ml; figure 3, panel C). However, this effect was remarkably pronounced with therapeutic concentrations of Imatinib (1 and 10 µM; 16.4±11.1 and 7.9±3.1 pg/ml, $p<0.02$) and Nilotinib (2 µM; 3.9±2.3 pg/ml, $p<0.05$), whereas it appeared less pronounced with therapeutic concentration of Dasatinib (50-100 nM, p=NS) and Ponatinib (10-50 nM, p=NS). TKIs induced a profound reduction of the concentrations of pro-inflammatory cytokines IL2, IFNγ and TNFα in supernatants (Figure 3, panels A, E-F), starting from the lowest drug concentrations; IL10 production was also strongly impaired by all the TKIs studied (Figure 3 panel D). On the contrary, the production of IL4 (Figure 3, panel B) was barely affected by TKIs (except Ponatinib). Taken together these data: 1) show that all the tested TKIs exert common anti-inflammatory effects; 2) suggest that, at therapeutic concentrations, some TKIs (i.e. Imatinib and Nilotinib) down-regulate pro-inflammatory Th17 cells activity more efficiently than others (i.e. Dasatinib and Ponatinib).

In vivo effects of Nilotinib on T cell subpopulations of patient with cGVHD

To assess whether the *in vitro* effects of TKIs on lymphocyte phenotype and function can also be observed in an *in vivo* setting, we evaluated 15 patients affected by steroid-dependent or steroid-refractory cGVHD that underwent treatment with Nilotinib in the setting of a phase 1-2 prospective multicenter study (see Material and Methods). Eight out of the 15 patients were still taking steroids and seven were treated with cyclosporine (2 mg/kg) when Nilotinib was started. We have previously shown [28] that mean plasma concentrations of Nilotinib was 817 ng/ml (±450) in all patients (corresponding to 1.6 µM ±0.9). Mean plasma trough level at the steady-state was 1.4 µM at 200 mg/die (range 0.4-2.1), 2.0 µM at 300 mg/die (range 0.6-5.0), 1.2 µM at 400 mg/die (range 0.1-2.1) and 4.7 µM for 600 mg/die (1 patient only). Figure 4 shows the overall frequencies of T, B and NK lymphocyte subsets in cGVHD patients receiving Nilotinib; no significant changes were observed in T and B lymphocyte proportions, irrespective of treatment schedule (not shown). NK cells proportions were also substantially preserved (Figure 4); in fact, a significant decrease of this subset was only observed when the highest dose of Nilotinib was given for 180 consecutive days (not shown). Collectively, these finding confirm the data observed *in vitro* and suggest that in patients with cGVHD the administration of Nilotinib does not induce relevant changes of the relative proportions of the main lymphocyte subpopulations, at least at the lowest doses.

In vivo effects of Nilotinib on T cell cytokine production

To investigate the impact of Nilotinib on cytokine network *in vivo*, we measured the cytokine concentrations in thawed plasma samples collected from Nilotinib-treated patients, as described in “Materials and Methods” section. As shown in figure 5, the administration of Nilotinib was associated with strong decrease in the production of pro-inflammatory cytokines, IL2 and IFNγ. IL10, TNFα levels and IL17 were also reduced in patients’ plasma samples following therapy,

although the latter two were mainly affected by longer duration of treatment (180 days) and by administration of higher drug doses (400 mg/daily). On the contrary, Nilotinib did not induce relevant changes in the production of IL4. These data confirm those generated *in vitro*, further suggesting that treatment with Nilotinib is associated with a consistent reduction of pro-inflammatory cytokines production, including IL17.

Discussion

To date, no standard treatment is available for SR-cGVHD [13]. Donor derived CD4 and CD8-positive T lymphocytes have been considered important effectors mediating cGVHD pathogenesis, although recent studies suggest that B cells might also play a role [8,29-30]. *In vitro* data suggest that TKIs strongly modulate both T- and B-cell mediated immune responses [25,31] while preliminary clinical data suggest a promising efficacy of both Imatinib and Nilotinib on SR-cGVHD [32]. In the present study, we first investigated the *in vitro* effects of four different TKIs (Nilotinib, Dasatinib, Imatinib, Ponatinib) on lymphocyte numbers and function. We observed heterogeneity in lymphocyte subsets susceptibility, according to the kind and concentration of TKI evaluated. Overall, T cell frequencies were not consistently affected by therapeutic doses of TKIs, whereas the proportions of B and NK cell showed a robust and linear decline, following exposure to increasing amounts of all the TKIs, except Nilotinib.

Clinical studies have linked NK cells to outcome following stem cell transplant (SCT); indeed, patients receiving SCT bearing high numbers of NK cells are less likely to develop acute and cGVHD [33,34]. These findings are consistent with mice models showing that donor NK cells can eradicate recipient antigen presenting cells (APCs) thereby preventing GVHD [35]. Among the different TKIs tested in the present study, only Nilotinib could preserve NK cells ($p<0.01$), thus suggesting that the immune modulatory effect of this drug may be more balanced toward an efficient control of GVHD compared to other TKIs. However, Nilotinib did not exert any significant effect on B cells, recognized as important players in the pathogenesis of cGVHD. [8].

In preclinical murine transplant models Tregs are able to prevent alloreactive T cell responses and experimentally induced GVHD, and recent studies suggest a role for Tregs in the protection from

both acute and cGVHD. [36-38]. In our model, whereas *in vitro* exposure to increasing doses of Imatinib, Dasatinib and Ponatinib induced a progressive and significant decrease ($p<0.01$) of total Tregs, Nilotinib reduced the frequency of this subpopulation only at concentrations exceeding 2 µM ($p<0.01$), i.e. above those usually achieved *in vivo*. This finding is in line with previously published data, pointing out a possible relationship between Tregs kinetics and Nilotinib dosage. [39]. It has been recently showed that high frequencies of naïve regulatory T cells are associated with lower risk of acute GVHD following haploidentical or sibling identical SCT [40], thus suggesting that different Treg subsets can exert a different impact on the outcome of SCT. The protective role of naïve T regulatory cell subset has also been showed in other clinical condition, such as pregnancy-associated pre-eclampsia, preterm delivery and gestational diabetes mellitus [41-42]. Of note, in the present study the frequency of naïve T regulatory cells increased following exposure to all the TKIs tested, suggesting that despite inducing the overall reduction of the total T regulatory cell pool, these drugs may indeed lead to the accumulation of T regulatory subsets provided with high immune modulating activity against GVHD.

Cytokine production is a key process by which activated T cells exert their immune responses and is involved in development and evolution of conditions characterized by immune hyper-activation, such as GVHD and transplant rejection. Of note, in the present study pro-inflammatory cytokine concentrations (including IL17) decreased significantly in culture supernatants following exposure to all the TKIs tested.

Overall, the results from our *in vitro* experiments suggest a peculiar and balanced activity of Nilotinib on lymphocyte's immune modulation: like other TKIs, this drug interacts with innate and adaptive immune response, showing anti-inflammatory properties; however, unlike other TKIs,

Nilotinib is able to better preserve cell subsets that have been shown to prevent the development of immune responses associated with GVHD, such as Treg and NK cells.

Based on these *in vitro* data, we further address the effect of Nilotinib on lymphocyte subpopulations and function in an *in vivo* setting, by analyzing blood samples from 15 patients affected by steroid-refractory cGVHD, after 90 or 180 days of therapy. To our knowledge, this is the first study addressing simultaneously both *in vitro* and *in vivo* effects of Nilotinib by the same experimental approach. Overall, the *in vivo* data confirm those obtained *in vitro*, showing that Nilotinib: 1) induces a consistent reduction of pro-inflammatory cytokines production, particularly IL2 and IFNγ: 2) substantially preserves lymphocyte subpopulations frequencies.

In addition, Nilotinib did not affect significantly Tregs frequency, although the phenotypic definition of this subpopulation *in vivo* did not include FoxP3 and CD127 (which were instead included in the phenotypic evaluation of this same subset *in vitro*), due to limited amount of samples available and to technical issues related to intracellular staining of thawed cells; adequate studies are needed to better define the kinetics of T regulatory cells following exposure to TKIs *in vivo.* The experiments were conducted on thawed samples, therefore the impact of Nilotinib on absolute numbers deserve further investigation. Compared to the *in vitro* findings, the *in vivo* administration of Nilotinib induced a slight, non-significant decrease of IL17 in plasma sample. The reasons of this discrepancy are unclear; however, we cannot exclude that this discrepancy could be related to differences between the kind of samples used for *in vitro* and *in vivo* studies; the former were in fact obtained from healthy donors, whereas the latter were collected from individuals exposed to different immunosuppressant drugs including steroids, and possibly affected by co-morbidities requiring additional therapy (i.e. anti-inflammatory drugs, antimicrobials, etcetera). Collectively, our data support the use of TKIs, Nilotinib in particular, in

549 patients developing cGVHD following SCT, and emphasize the broad and potent anti-inflammatory
550 effect of Nilotinib and other TKIs, supported by the robust decline of all the pro-inflammatory
551 cytokines included in the analysis. Further studies are necessary to better clarify the clinical impact
552 and safety of different TKIs in this clinical setting; in particular, additional investigation by
553 adequately powered clinical trials is required to understand whether the experimental data
554 provided by this study will translate into clinical benefits for patient affected by steroid refractory
555 cGVHD. As cGVHD can involve many different organs such as skin, liver and lungs, further studies
556 are also needed to address the therapeutic effectiveness of Nilotinib (and other TKIs) on different
557 body districts. Moreover, the negative (although not significant at least for Nilotinib) impact of
558 TKIs on the total T regulatory cell pool frequencies could suggest a possible association of TKIs
559 with other treatments aimed to increase the Treg populations (e.g. IL2 low dose or extracorporeal
560 photopheresis) [43-44] in order to increase the response rate in these patients. Finally, the role of
561 different T regulatory subset in controlling cGVHD following SCT should be further addressed, as
562 the accumulation of naïve Tregs may impact more favorably than other subsets on the prevention
563 of cGVHD development.

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Figure 1 – Changes in T regulatory cells frequencies and death rate proportions following exposure to TKIs *in vitro*

Lymphocytes were gated by forward scatter (FSC) and side scatter (SSC) profile. T regulatory cells were identified by the co-expression of CD3, CD4, CD25 surface markers and FoxP3 intracellular marker (line plots, panels A-D); dying cells were identified by the expression of the marker 7AAD on the surface of CD3, CD4 and CD25-positive cells (bar plots, panels E-H). Results are expressed as percentages on total CD25-positive cells. Means and standard deviations are reported; *p<0.05; **p<0.02.

Figure 2 – Naive T regulatory cells frequencies following exposure to TKIs *in vitro*

Lymphocytes were gated by forward scatter (FSC) and side scatter (SSC) profile. Naïve T regulatory cells were identified by the expression of the marker CD45RA on the surface of CD3, CD4, CD25 and FoxP3-positive cells (line plots, panels A-D). Results are expressed as percentages on total T regulatory cells. Means and standard deviations are reported; *p<0.05; **p<0.02.

Figure 3 – T cell production of pro-inflammatory and immuno-modulatory cytokines following exposure to TKIs *in vitro*

T cells were purified from PBMCs through positive selection by magnetic cell separation CD3 microbeads and cultured. Cytokine production was measured on cell culture supernatants; baseline values (pg/ml) are compared to results obtained following exposure of cells to increasing concentrations of each TKI. Means and standard deviations are reported; *p<0.05; **p<0.02.

Figure 4 – Lymphocyte subpopulations changes following exposure to Nilotinib *in vivo*

PBMCs obtained from SR-cGVHD affected patients treated with Nilotinib at different dose schedule were thawed, labeled with monoclonal antibodies and examined by flow cytometry. Frequencies of T, B and NK subset are shown. Average values and standard deviation at baseline (white bars) and following 90 days (light gray bars) and 180 days (dark grey bars) of therapy are reported. Values are expressed as percentage of total lymphocytes; changes induced by different Nilotinib dose schedules are represented together.

Figure 5– Modification of cytokine production following exposure to Nilotinib *in vivo*

Cytokine concentrations were determined on plasma samples from Nilotinib-treated patients after thawing. Average values and standard deviation at baseline (white bars) and following 90 days (light gray bars) and 180 days (dark grey bars) of therapy are reported. Changes induced by different Nilotinib dose schedules are represented together. *p<0.05; **p<0.02.

Supplementary figure S1 – T helper cells frequencies and death rate proportions following exposure to TKIs *in vitro*

Lymphocytes were gated by forward scatter (FSC) and side scatter (SSC) profile. Changes of CD4-positive T cell frequencies (line plots, panels A-D) and death rates (bar plots, panels E-H) following exposure to different TKIs are represented. Means and standard deviations are reported; *p<0.05; **p<0.02.

Supplementary figure S2 – T cytotoxic cells frequencies and death rate proportions following exposure to TKIs *in vitro*

Lymphocytes were gated by forward scatter (FSC) and side scatter (SSC) profile. T cytotoxic cells were identified as CD3-positive and CD4-negative lymphocytes. Changes of cell

739 frequencies (line plots, panels A-D) and death rates (bar plots, panels E-H) following exposure
740 to different TKIs are represented. Means and standard deviations are reported; *p<0.05;
741 **p<0.02.
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Table 1 – Lymphocyte subpopulations changes following exposure to increasing TKIs concentrations

	TKI concentration	T lymphocytes (%)	B lymphocytes (%)	NK cells (%)	T lymphocytes death rate (%)	B lymphocytes death rate (%)	NK cells death rate (%)
	baseline	**75.9 ± 3.8 3**	**9.1 ± 2.02**	**7.2 ± 0.65**	**0.1 ± 0.05**	**0.0**	**0.7 ± 0.25**
IMATINIB	1 µM	81.1 ± 4.17	7.4 ± 3.01	6.2 ± 1.47	0.3 ± 0.08**	0.02 ± 0.04	0.9 ± 0.28
	10 µM	84.5 ± 1.79	5.7 ± 2.18*	6.1 ± 1.24*	0.4 ± 0.04**	0.04 ± 0.05	1.3 ± 0.37*
	50 µM	78.2 ± 6.38	4.6 ± 1.30**	2.5 ± 0.87*	1.9 ± 0.58 **	0.7 ± 0.11*	3.5 ± 1.61**
NILOTINIB	0,5 µM	76.9 ± 2.37	8.7 ± 2.79	6.6 ± 0.97	0.2 ± 0.15	0.04 ± 0.09	1.0 ± 0.30
	1 µM	73.5 ± 4.02	8.7 ± 4.81	6.2 ± 1.23	0.2 ± 0.10	0.3 ± 0.29	1.2 ± 0.32
	2 µM	79.9 ± 3.79	8.5 ± 2.58	5.8 ± 1.79	0.3 ± 0.11*	0.1 ± 0.22	1.0 ± 0.43
	10 µM	77.5 ± 3.48	8.6 ± 2.53	5.5 ± 1.54	0.4 ± 0.16*	0.1 ± 0.22	1.0 ± 0.32
DASATINIB	50 nM	76.8 ± 2.81	3.4 ± 0.77**	3.2 ± 0.70**	1.3 ± 0.71*	0.2 ± 0.17*	1.5 ± 0.38*
	100 nM	71.8 ± 3.01	3.5 ± 1.06**	2.4 ± 0.83**	2.8 ± 0.87**	0.9 ± 0.36**	3.1 ± 0.50**
	200 nM	0.0**	0.0**	0.0**	100 ± 0.00**	100 ± 0.00**	100 ± 0.00**
PONATINIB	10 nM	81.7 ± 5.41	3.5 ± 0.92**	5.0 ± 0.39**	0.7 ± 0.13**	0.1 ± 0.00*	2.2 ± 0.53**
	50 nM	88.2 ± 4.66	1.7 ± 0.65**	1.9 ± 0.76**	1.5 ± 0.22**	2.7 ± 2.05*	22.5 ± 3.47**
	100 nM	0.0**	0.0**	0.0**	100 ± 0.00**	100 ± 0.00**	100 ± 0.00**

T, B and NK subpopulation frequencies and death rates are reported; baseline values are compared to results obtained following exposure of cultured cells to increasing concentrations of each TKI. Lymphocytes were gated by forward scatter (FSC) and side scatter (SSC) profile. T lymphocytes were identified as CD3-positive cells; B Lymphocytes were identified as CD3-negative CD19-positive cells; NK cells were identified as CD3-negative CD16 and CD56-double positive cells. Means and standard deviations are reported; *p<0.05; **p<0.02.

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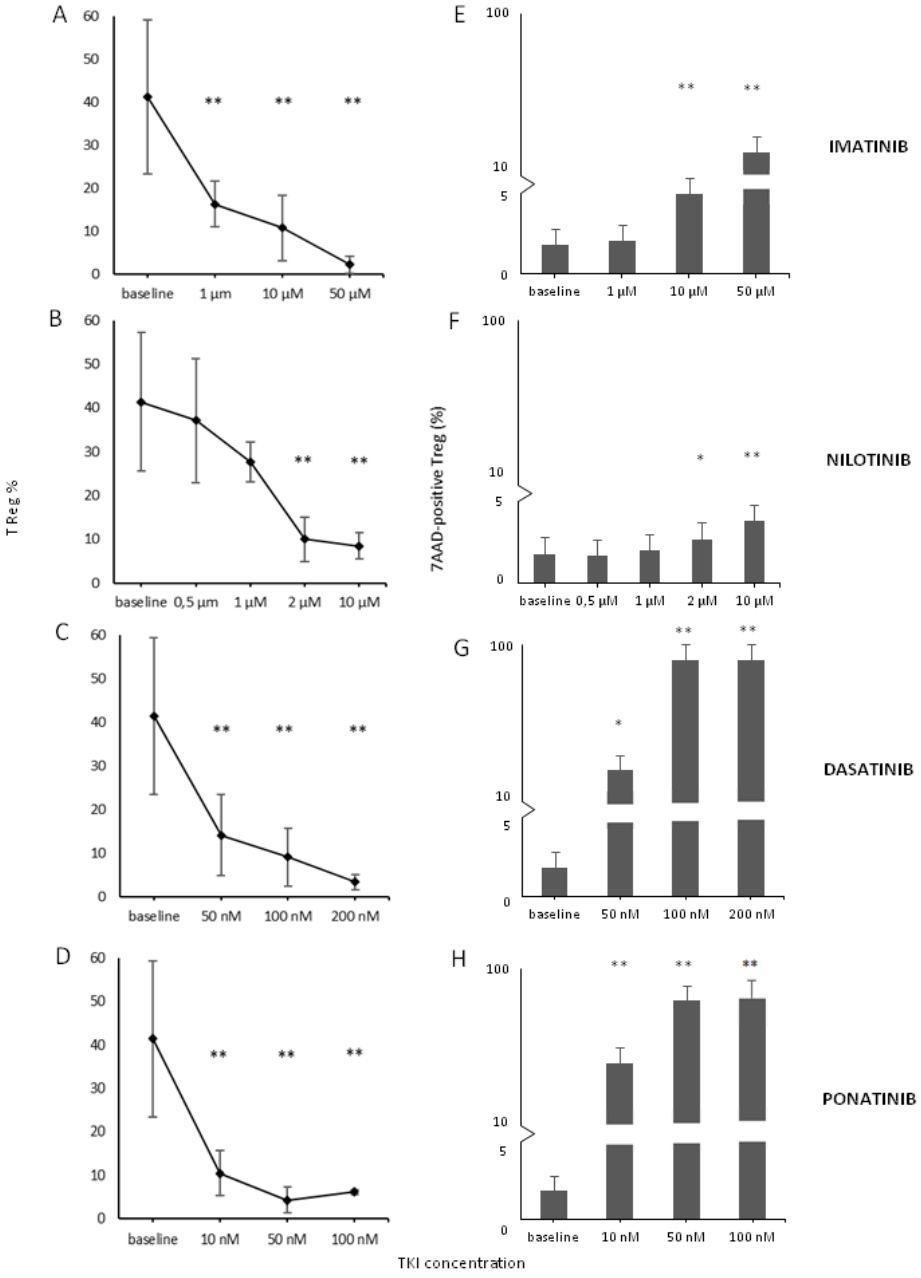

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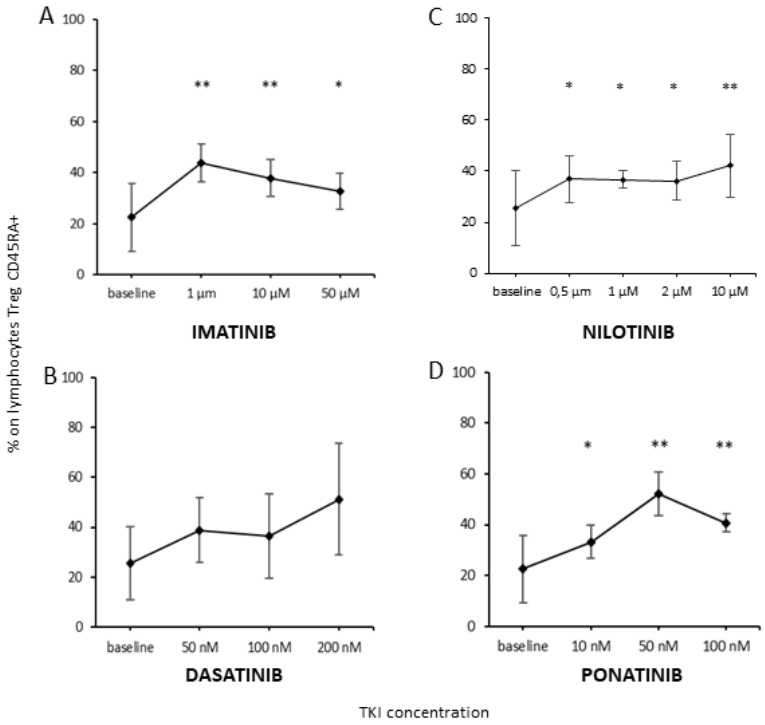

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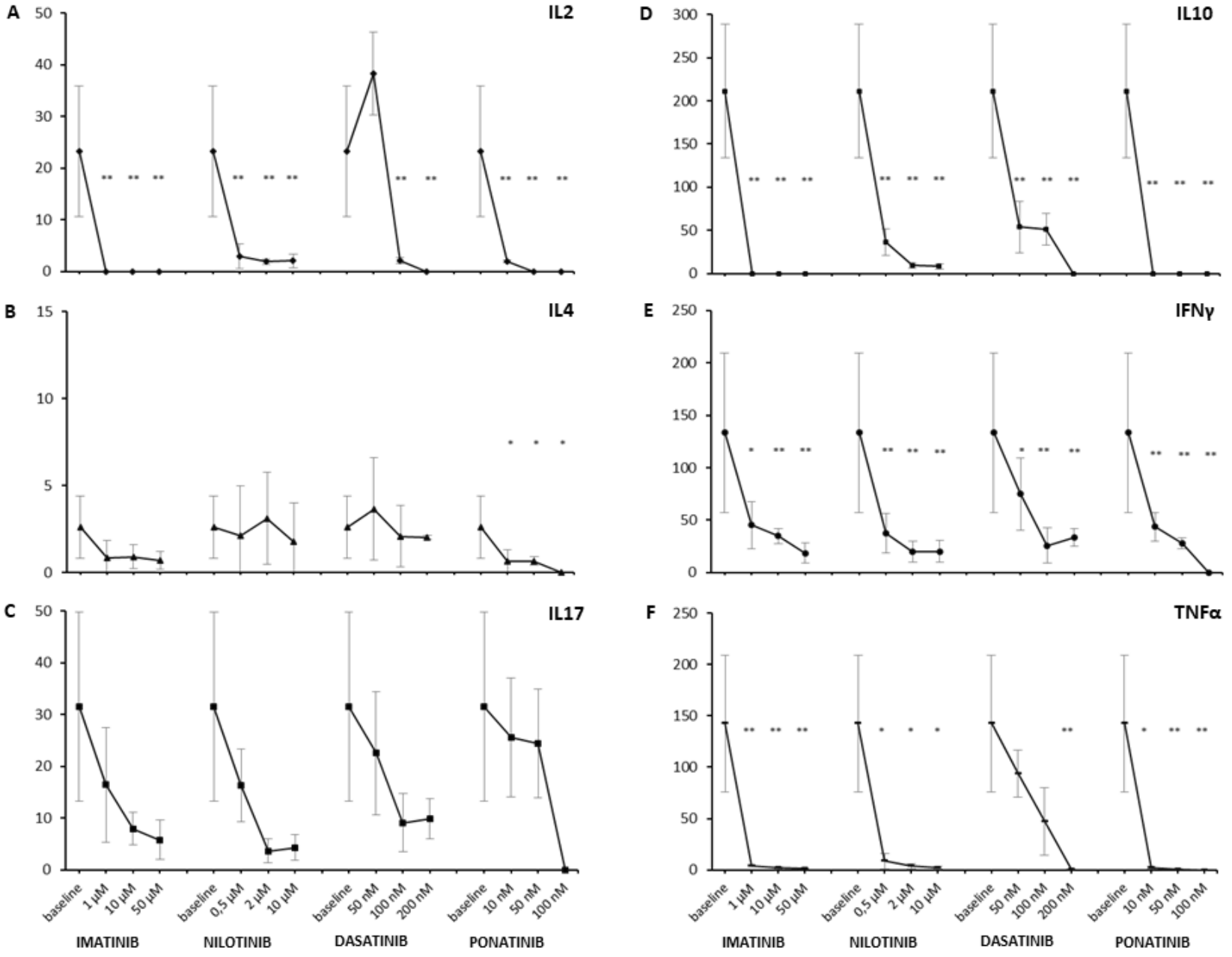
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IFNγ
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TNFα
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1 μM
10 μM
50 μM
0,5 μM
2 μM
10 μM
50 nM
100 nM
200 nM
10 nM
IMATINIB
NILOTINIB
DASATINIB
PONATINIB

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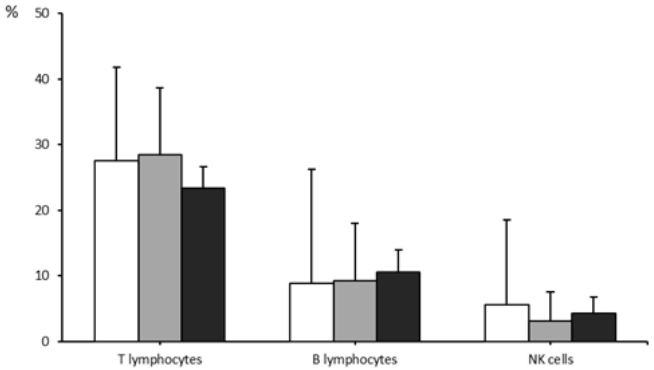

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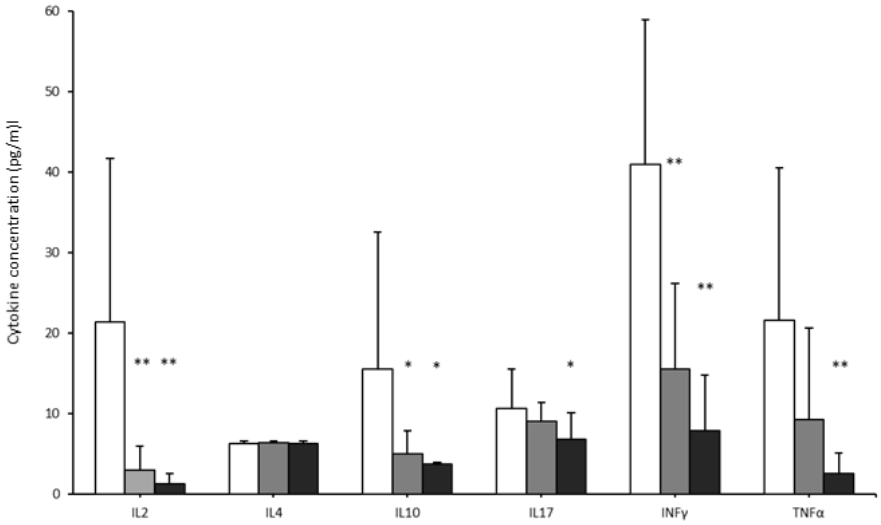

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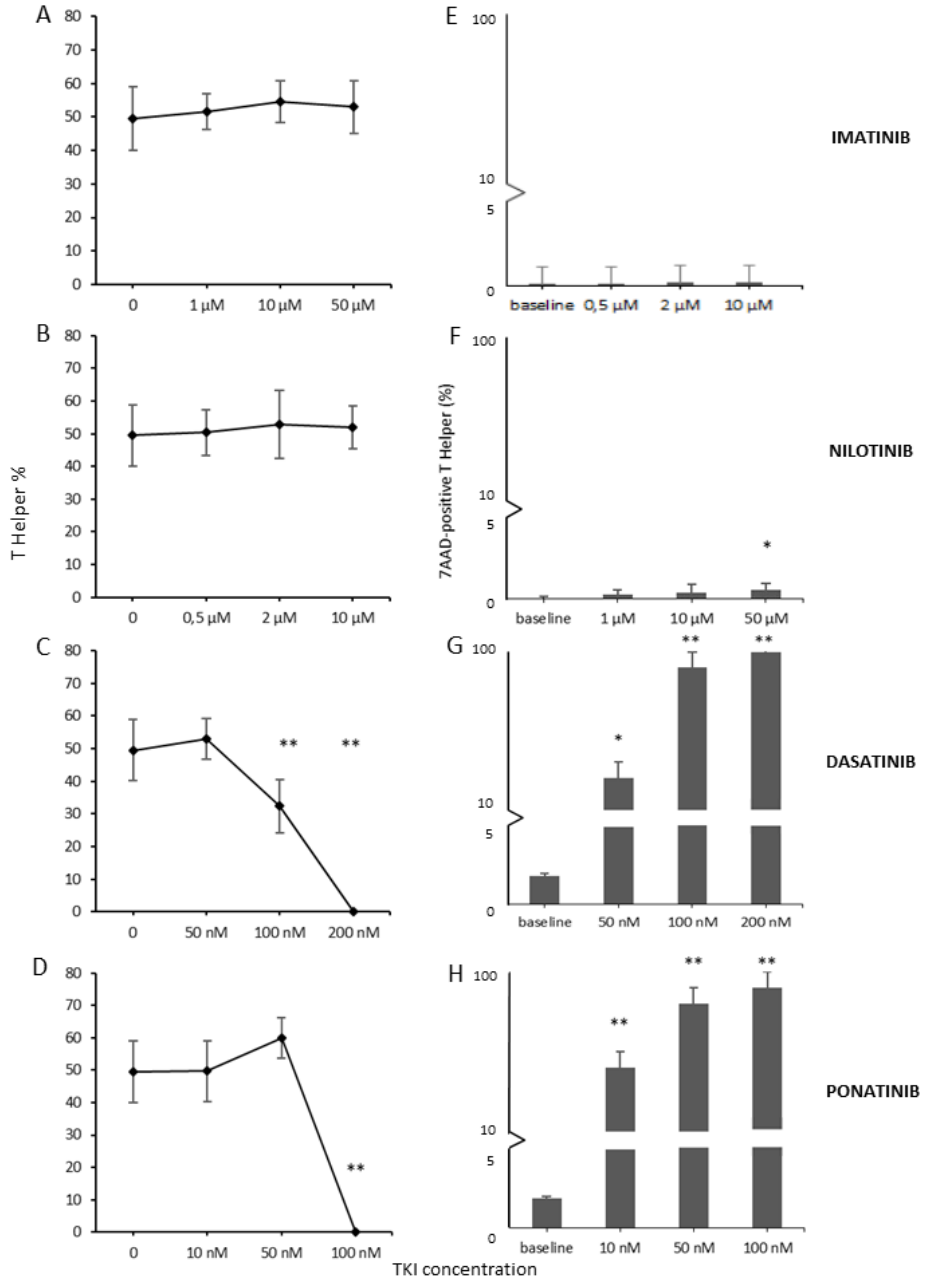

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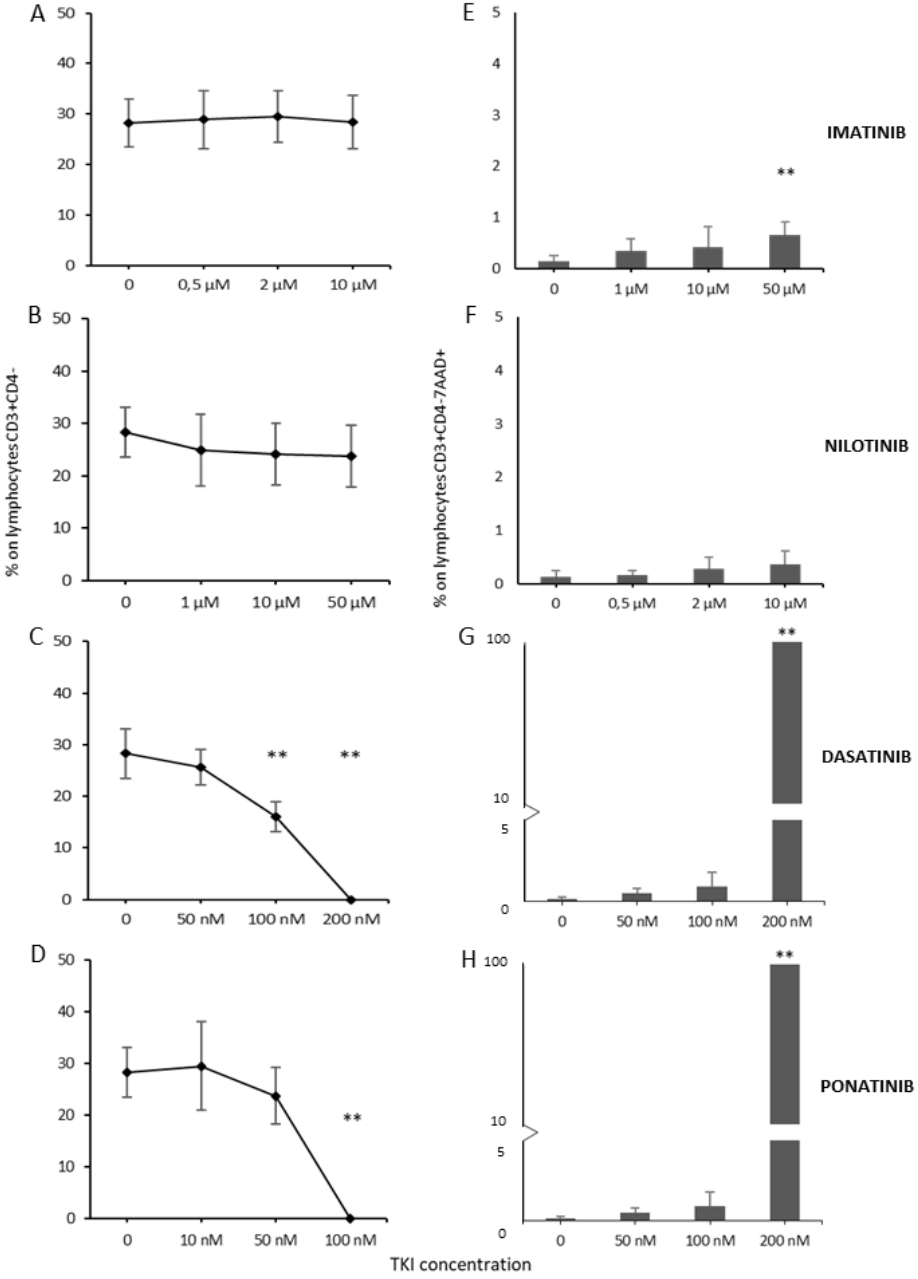

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